SYNOPSIS

The geometric organization of myofibers in the mammalian heart wall supports synchronous contraction and electrical conduction while also providing mechanical strength. The mammalian heart wall is densely packed with cardiomyocytes that are aligned end-to-end to form them (1). Myofiber malformations can result in pathological cardiac illnesses, including cardiomyopathy, remodelling after myocardial infarction, and electrical propagation (1). Myofibers have been described as a helical continuum on a coarse spatial scale, looping around the heart chambers (1,2). A three-layer model(3) and descriptions such as nested donuts, toroids and pretzels(4), and a single helical band are among the competing models of fiber organization that still exist (5). Despite the lack of an agreed-upon model, the current heart wall fiber geometry models used in cardiovascular research are primarily obtained from millimeter-resolution diffusion-tensor magnetic resonance imaging (DT-MRI)(4,6–9). These models consistently support a smooth counter clockwise rotation of the heart wall fibers in transmural penetration and, by definition, forbid singularities in fiber orientation. There are also myofiber orientation rule-based models and minimal surface models that are generally in line with the DT capabilities of MRI (10). In addition, statistical atlases of fiber geometry derived from imaging of numerous participants were obtained using DT-MRI (11,12). While these models can progress research on the electrical and mechanical functions of the heart wall (13), they all have issues with spatial resolution because, at this scale, hundreds of cardiomyocytes can fill a single voxel.

Micron-scale light microscopy allows the recovery of myofiber shape at the resolution of individual cardiomyocytes. However, studies in this area have so far concentrated on thin sections of heart tissue (11) or 3D stacks of heart tissue without determining myofiber geometry (15,16), or based on histological sections that are typically only suitable for two-dimensional imaging (17,18). Recent research has not recovered myofiber or cardiomyocyte orientation at the whole-heart scale. Remarkably, the geometry and orientation of cardiomyocytes with respect to myofibers at the micron scale remain unknown.

To address this fundamental issue, we integrated computer vision methods with deep and wide imaging based on confocal light microscopy. Using these two techniques, we were able to determine the micron-scale orientations of cardiomyocytes over the whole

long- and short-axis ventricular regions of mouse hearts. Our three-dimensional reconstructions at exceptional spatial resolution uncovers previously unreported long-axis bands of fibers at the outer ventricle walls and are distinct from the helical or circumferential myofibers. This finding marks a conceptual advance in understanding the architecture of myofibers of the heart. Virtually all computational models of heart-wall contraction or electrical wave propogation do not include the unique long-axis outer-wall fiber structure. Its involvement could significantly improve our understanding of heart wall mechanics and electrical conduction and create new avenues for research on disorders associated with changes in fiber organization at different spatial scales in the heart wall tissue.

The current study titled '**Towards a cellular architecture of three dimensional array of muscle fibers'** is divided into six chapters.

Chapter 1 outlines and reviews the literature on heart wall geometry and open questions. Chapter 2 describes the Objectives and experimental approach of the study. Integrating fluorescence-based micron-scale heart imaging, geometric image analysis, and myofiber reconstruction methods for microscopic images is a crucial advancement in our study. This combination led to a resolution gain of three orders of magnitude in scale (i.e., from millimeters to microns), revealing an entirely new fiber system that has escaped discovery by previous lower-resolution imaging methods. A detailed description of our pipeline and findings will be highlighted in Chapters 3 and 4. The results of this study have been communicated and are currently under submission. A preprint version of this manuscript titled 'Myofiber reconstruction at micron scale reveals longitudinal bands in heart ventricular walls'available at the biorXiv repository. The current limitations of this study are explained in Chapter 5. Our cardiomyocyte orientation reconstructions from thick heart tissue sections can lay the foundation for future pipeline establishment for cellular-scale imaging of cleared intact mouse hearts in their native context and disease states. Chapter 6 discusses these future directions and our attributes for the present understanding of heart wall mechanics, adding implications for studying diseases related to myofiber disorganization.

Motivation for this thesis

Given the crucial role that myofiber organization of the heart wall plays in regulating electrical conduction and dynamics of the heart wall, recovering cardiomyocyte and myofiber geometry at the cellular level is of utmost importance. The majority of available models, which function at the millimeter scale, show a continuous network of helical fibers rotating from the outer to inner walls in a clockwise orientation; however, they are unable to arrive at a common model. The present heart wall models, primarily developed from DT-MRI and millimeter-scale imaging, are insufficient because they may fit hundreds of cardiomyocytes in a single voxel and lack cellular-scale information. We sought to reproduce the intricate geometry of myocytes and myofibers in the ventricular walls at the micron scale using confocal microscopy and computer vision techniques. This discovery could make essential advancements in our understanding of the structure and operation of the heart walls.

Chapter 2: Objectives

The primary objective of this thesis is to elucidate the 3D architecture of cardiomyocyte orientation for intact ventricular heart tissue at a micron-meter scale resolution. The output from this work will enable us to comprehend the blueprint for the cellular architecture of a wild-type heart, as it is controlled by cardiomyocytes. This can be applied to understand the modifications in cardiomyocyte orientation induced by dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM).

The specific objectives of this study are detailed below.

1. Establish a pipeline for tissue preparation for deep imaging using confocal microscopy.
2. Integrative approaches for deep tissue image analysis and estimation of myofiber orientation.
3. 3D reconstruction of myofiber orientation using tractography at cellular/micron scale.

Chapter 3: Methods and Materials

Sample preparation and imaging:

We obtained mouse and rat hearts from wild-type female C57BL/6 and Wistar strains, respectively. All cardiac samples were perfused with 1X PBS that was heparinized to prevent blood clots. We used a modified CLARITY technique to clear heart tissue (19).As illustrated in Fig. 1A, cleared mouse hearts, uncleared fixed mouse hearts, and rat hearts were sectioned into short- and long-axis sections using Compresstome VF-300. For labeling, we used wheat germ agglutinin (WGA, W21404, Thermo Fisher) conjugated to Alexa Fluor 633 at 150 µg/ml. The heart tissues were stained with WGA and then placed in RIMS imaging solution (88 percent Histodenz, Sigma D2158 in 20mM Phosphate buffer, pH 7.5). The cardiac tissues were then mounted with a spacer and two coverslips of 170µm thickness. Confocal images were taken using an Olympus FV3000 confocal microscope coupled with Olympus PlanApo 1.25X and Olympus UCPLFN 20X CorrM32 85 mm scale air objectives (NA=0.73). A lower magnification image that covered the entire tissue block area was created for each section using a 1.25X objective. Olympus FluoView^{TM} software was used to map the field of view. 320x320 pixel fields of view with isometric voxel sizes of 1.98 μm^3 was used with a 20X objective for micron-scale imaging. Image stacks with maximum depths of 300 µm and 50 µm were acquired for cleaned and uncleared heart tissues. The sample was excited with a 640 nm laser line, and an emission between 650 and 670 nm was recorded.

Pre-processing of heart tissue image stacks:

We employed iterative Richardson-Lucy deconvolution along with total variation regularization for deconvolution to deal with the nonideal point spread function (PSF). Unsupervised denoising was applied to deconvolved image stacks using a dictionary learning technique. The overlapping fields of view were tiled using phase correlation in the Fourier domain between the adjacent fields of view.

Estimation of cell orientation using structure tensor and myofiber reconstruction:

The intensity gradient of the WGA stain, which was absorbed by cell membranes (Fig. 1B) was used to calculate myocyte orientation. We computed the structure tensor for each voxel in the stack of the deconvolved and denoised images. To capture the long-axis orientation of myocytes, we assigned the orientation in the direction where the intensity fluctuated the least to each voxel. To verify their validity, we compared the structure tensor-based predictions of cell orientations with the ground-truth orientations of many manually segmented myocytes. To illustrate the angular changes, we employed

a 3-dimensional array of pivoting cylinders and streamlines, or "orientation glyphs," to visualize the parula-color-coded orientation field (Fig. 1B).

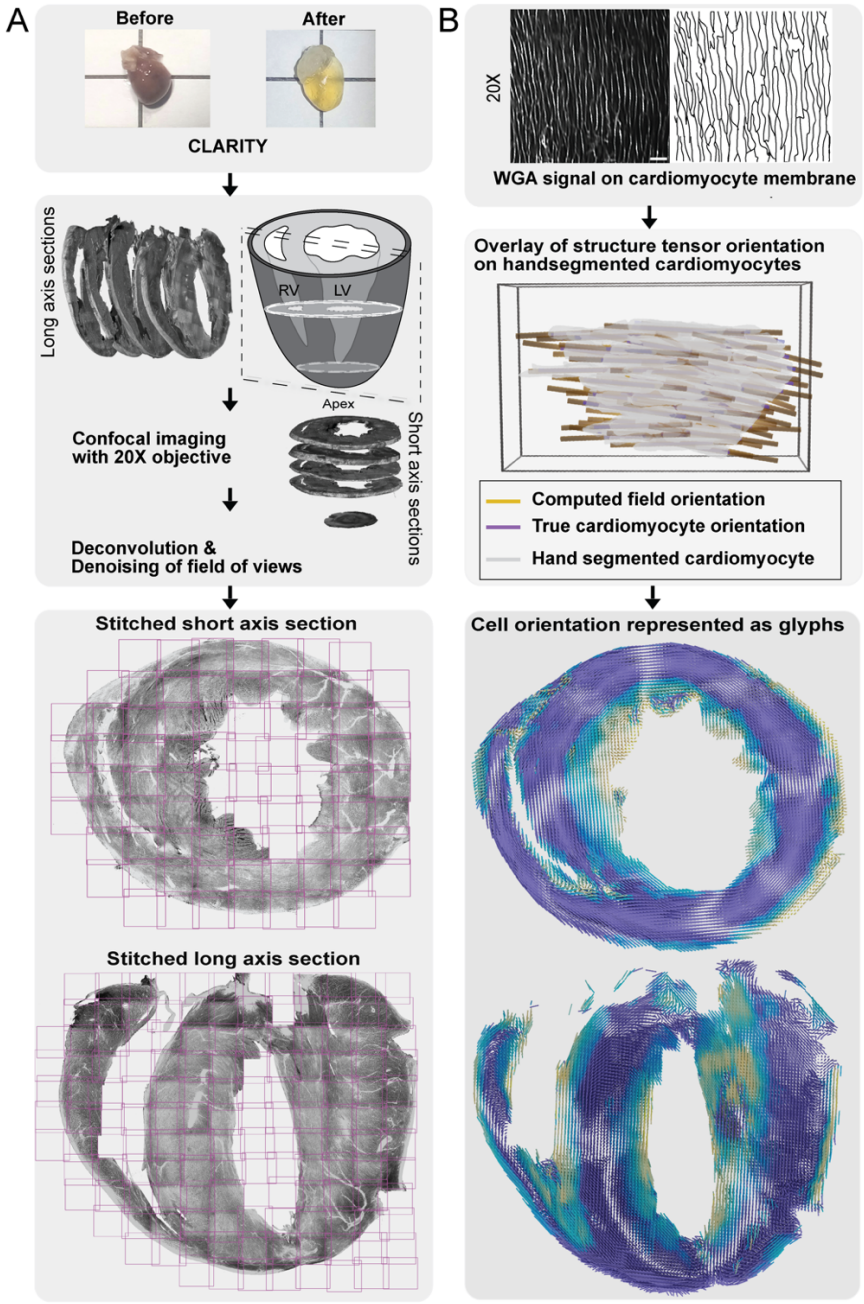

Figure 11: Scheme for tissue preparation, imaging, and computational analysis. **A.** Flowchart illustrating the tissue preparation, imaging, and pre-processing pipeline. The top row depicts representative images for clearing (CLARITY) of the entire heart tissue. The middle row shows the tissue sections obtained in different orientations. The bottom panel shows the generation of a composite tiled image from the acquired field of views. **B.** A flowchart illustrating the computational pipeline for estimation and orientation reconstructions of myofibers. The top row shows the features of cardiomyocytes picked up by WGA staining intensity gradient with respect to the cell membrane. The middle row shows the orientation estimation from many hand-segmented cardiomyocytes, which is computationally estimated. This ground-truth experiment validated the

computational pipeline of the orientation estimation as an independent experiment. The bottom row shows the operations performed on the stitched composite image.

Chapter 4: Results and Conclusions

The heart pumps blood and functions because of the synchronized contraction of cardiomyocytes. A consensus model for myofiber geometry does not yet exist because of the limited spatial resolution of whole-heart imaging methods and the fragmented nature of histological studies. We provide the first three-dimensional reconstruction of myofibers across the entire mouse ventricular wall at micron-scale resolution. In the outer ventricular wall region, our micron-scale imaging analysis revealed prominent and distinct longitudinal myofiber bands orthogonal to the well-known circumferential bands in the middle wall.

Chapter 5: Limitations

Mouse ventricular heart regions were the primary subjects of this thesis. Conclusions on myocyte orientation in the atrial or basal ventricular areas or other muscle systems were not addressed in this thesis; hence, the details of myocyte orientation in the atrium, basal regions, or other muscle systems will not be included. This longitudinal outer wall system's functional role in regulating heart contractions is still up for question. Modeling biophysical characteristics and cellular variations between circumferential and longitudinal myofiber connections must be explored.

Chapter 6: Discussion

Our imaging revealed the presence of a fiber system similar to that in rat hearts. Similar fiber patterns have been reported in other organs and the entire animal body, indicating that this unusual longitudinal fiber geometry is not exclusive to the heart (20,21) .The smooth muscle peristaltic motions found in the esophagus and intestinal tubes are known to depend on the coexistence of circumferential and longitudinal fiber systems at the organ level(20). However, the orthogonal arrays in these layered muscle fiber structures occupy an equivalent volume, which is a significant distinction between them and the heart .

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